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Loss of olfaction in sea snakes provides new perspectives on the aquatic adaptation of amniotes

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Abstract

Marine amniotes, a polyphyletic group, provide an excellent opportunity for studying convergent evolution. Their sense of smell tends to degenerate, but this process has not been explored by comparing fully-aquatic species with their amphibious relatives in an evolutionary context. Here, we sequenced the genomes of fully-aquatic and amphibious sea snakes, and identified repertoires of chemosensory receptor genes involved in olfaction. Snakes possess large numbers of the *olfactory receptor* (*OR*) genes and the *type-2 vomeronasal receptor* (*V2R*) genes, and expression profiling in the olfactory tissues suggests that snakes use the ORs in the main olfactory system (MOS) and the V2Rs in the vomeronasal system (VNS). The number of *OR* genes has decreased in sea snakes, and fully-aquatic species lost the MOS which is responsible for detecting airborne odors. In contrast, sea snakes including fully-aquatic species retain a number of *V2R* genes and a well-developed VNS for smelling underwater. This study suggests that the sense of smell also degenerated in sea snakes, particularly in fully-aquatic species, but their residual olfactory capability is distinct from that of other fully-aquatic amniotes. Amphibious species show an intermediate status between terrestrial and fully-aquatic snakes, implying their importance in understanding the process of aquatic adaptation.

BACKGROUND

Shifts between terrestrial and aquatic lifestyles are among the most striking types of evolutionary transitions in the history of life. Vertebrates invaded land during the Devonian to the Carboniferous in two steps: first, they became amphibious (*i.e.*, both aquatic and terrestrial habitats are required) with the emergence of tetrapods, and then they adapted for terrestriality with the emergence of amniotes [1]. Among groups containing mostly terrestrial amniotes, there are several groups which re-adapted to the aquatic habitat independently from each other. Amniotes are also suggested to have re-invaded water from land with two major steps: they become amphibious prior to the completion of aquatic invasion. For example, all extant cetaceans are fully-aquatic but their intermediate ancestors from the Early Eocene were amphibious [2] (Fig. 1). Marine elapids (Suborder Serpentes, Order Squamata, Class Reptilia), collectively called sea snakes, consist of two monophyletic clades, Laticaudini and Hydrophiini. Laticaudins are oviparous and lay eggs on land, whereas hydrophiins are viviparous and spend all their life in water. Both groups have a paddle-shaped tail adapted to aquatic locomotion, but laticaudins retain enlarged ventrals required for terrestrial locomotion which hydrophiins lost [3]. Although recent studies suggested that laticaudins and hydrophiins adapted to the marine habitat independently, these two clades are phylogenetically close to each other with a divergence time of approx. 12–20 million years ago [4-7]. Thus, sea snakes provide an excellent study system of aquatic adaptation because phylogenetically closely related fully-aquatic and amphibious species can be compared directly.

Aquatic amniotes offer a valuable opportunity for studying convergent evolution because evolutionary hypotheses of specific adaptation can be tested for multiple aquatic groups that migrated from land to water independently to each other [8, 9]. One of the most remarkable differences between terrestrial and aquatic vertebrates involves the sense of smell. Broad taxa of

vertebrates detect odorants mainly using four major groups of G-protein coupled receptors (GPCRs) encoded by different multigene families: olfactory receptors (ORs), trace amine-associated receptors (TAARs) and two types of vomeronasal receptors (V1Rs and V2Rs) [10]. It has been hypothesized that olfactory GPCRs are functionally divided into two groups, receptors for airborne molecules and those for water-soluble molecules [10, 11]. The *OR* gene repertoire changed drastically in our ancestors during their transition from water to land, and amphibians show an intermediate form. Modern anurans retain mostly ancestral *OR* gene subfamilies for detecting water-soluble molecules which amniotes lost, but they also share newly diverged *OR* gene subfamilies with amniotes which are considered to detect airborne odorants [12-14]. Aquatic tadpoles (larvae of amphibians) possess olfactory organs for smelling underwater, but extreme remodeling occurs during metamorphosis to meet the requirement of the adult lifestyle, and adult anurans develop a so-called “air nose” for smelling in the air [15], in which the newly-diverged *OR* genes are expressed [16]. The *OR* genes possessed by terrestrial amniotes are prone to secondary loss from the genomes of aquatic amniotes [17-21], and extant toothed whales possess no olfactory nervous systems [22]. Baleen whales, the other group of extant cetaceans, still possess a functional olfactory system, but anatomical, histological and genomic studies suggest that they cannot smell underwater and their olfactory capability is highly limited [23-26]. However, olfactory capabilities of non-cetacean aquatic amniotes remain largely elusive. Furthermore, no extensive studies on genomes of amphibious amniotes have ever been compared and contrasted with those of fully-aquatic relatives, in spite of their importance upon aquatic adaptation. Here, we sequenced and assembled the genomes of fully-aquatic and amphibious sea snakes. Olfactory GPCR genes were identified in each genome assembly, and the expression profiling of these receptors was performed. Our present study explores the genomic traces of evolution of olfaction in sea snakes and provides new perspectives on the aquatic adaptation of amniotes.

RESULTS

Sea snake genome assemblies

We sequenced and assembled the genomes of two hydrophiins (*Hydrophis melanocephalus* and *Emydocephalus ijimae*) and two laticaudins (*Laticauda laticaudata* and *L. colubrina*). These genome assemblies are estimated to contain at least 90% of all protein-coding genes (including those recognized as 'Fragmented' by BUSCO [27, 28] or 'Partial' by CEGMA [29]) based on completeness assessments using an one-to-one reference ortholog set (Table S1).

Different methodologies were employed for performing *de novo* assembly of the genomes of four snakes (see Materials and Methods for detail). We assembled the genome of *L. colubrina* based on the linked-read sequencing technology [30, 31]. It is known that this method allows us to generate relatively long genome sequences of diploid species with a single library for short read sequencing [32]. Among the four species sequenced in this study, the *L. colubrina* assembly shows the largest scaffold N50 length (3.1 Mbp) and completeness score of one-to-one ortholog coverage (Table S1). However, the proportion of truncated genes in the *L. colubrina* genome assembly do not differ greatly in comparison with other assemblies (Table S2). Consistently, the contig N50 lengths do not largely vary between the assemblies of the four species (Table S1).

Olfactory GPCR gene repertoires in snake genomes

We identified the olfactory GPCR genes in the genome assemblies of sea snakes and their terrestrial relatives. Snakes possess large numbers of *OR*s and *V2R*s, which vary between

118 species (mean numbers of intact *OR*s and *V2R*s are 194 ± 136 and 240 ± 136 [mean ± standard
119 deviation, calculated using all snake species shown in Fig. 2], respectively), whereas the
120 numbers of *TAAR*s and *V1R*s are small and comparable across species (snakes possess only two
121 or three intact *TAAR*s and two intact *V1R*s, as described below) (Fig. 2).

122 Sea snakes possess a smaller number of intact *OR* genes with higher proportions of pseudogenes
123 (Table S2) compared with terrestrial snakes (mean number of intact *OR*s of hydrophiins: 63.5 ±
124 14.9, laticaudins: 114 ± 12.7, terrestrial snakes: 335 ± 40.8 [mean ± standard deviation]) mainly
125 due to massive loss of the *OR* genes in the sea snake lineages (Fig. 3A). They also possess a
126 relatively small number of intact *V2R*s, but the numbers of intact *V2R* genes vary greatly
127 between species (mean number of intact *V2R*s of hydrophiins: 137 ± 95, laticaudins: 177.5 ±
128 121, terrestrial snakes: 351 ± 104 [mean ± standard deviation]), and massive gain of the *V2R*
129 genes is observed in two species of sea snakes, *E. ijimae* and *L. colubrina* (Fig. 3B). Snakes
130 possess two intact *TAAR*s (*TAAR1* and *TAAR5*). In addition, terrestrial snakes and laticaudins
131 possess one more intact *TAAR* gene (*TAAR2*, which is pseudogenized in the hydrophiin
132 genomes). All snakes including sea snakes (except for the common viper) possess two intact
133 *V1R*s, the *ancV1R* [33] and a *V1R* gene which is not orthologous to the mammalian *V1R*s
134 (*Squamata-V1R*, Fig. S1).

135

136 **Expression of the olfactory GPCR genes**

137 There are two anatomically distinctive olfactory systems in terrestrial snakes, the main olfactory
138 system (MOS) and the vomeronasal system (VNS). The olfactory epithelium of the MOS (the
139 main olfactory epithelium) is located in the nasal cavity (NC), and that of the VNS (the
140 vomeronasal epithelium) is located in the vomeronasal organ (VNO) [34]. The snake tongue
141 also plays a role in the VNS by delivering chemicals to the VNO [34]. We performed

transcriptome sequencing with RNA-seq on these potential olfactory organs of *L. laticaudata* and *H. melanocephalus*. The expression pattern of these genes in *L. laticaudata* suggests that most of *OR*s are used in the MOS, while *V2R*s function in the VNS (Fig. 4). It is noted that some pseudogenes are also expressed in the olfactory organs, coinciding with previous reports (e.g. Zhang et al. [35]). Expression levels of *V1R*s and *TAAR*s suggest that the *ancV1R* is expressed in the VNO of both species, and the *TAAR2*, which is pseudogenized in the hydrophiin genomes, is expressed in the NC of *L. laticaudata* (Table S3).

An intact *OR* gene is expressed in the tongue in each snake species (Fig. 4, indicated by arrows). The arrowed gene of *Hydrophis* and that of *Laticauda* are orthologous to each other. All squamates investigated in this study (except for *Emydocephalus*) possess one-to-one orthologues of this *OR* gene (Fig. S2).

DISCUSSION

Evolution of the main olfactory system

The repertoires and expression pattern of the olfactory GPCR genes shown in this study suggest that snakes mainly use *OR*s in the MOS and *V2R*s in the VNS. It has been widely considered that the VNS is the predominant chemosensory system in snakes, being more responsible than the MOS for their sense of smell [34, 36]. However, in our results, the numbers of intact *OR*s and *V2R*s are almost comparable among terrestrial snakes (Fig. 2), implying that terrestrial snakes potentially detect and discriminate as many chemicals using the MOS as using the VNS.

Sea snakes possess an apparently smaller number of intact *OR* genes compared with terrestrial snakes, and our phylogenetic analysis suggests that it is mainly because of massive loss of the *OR* genes in the sea snake lineages after the king cobra–sea snakes split (Fig. 3A). Although the most recent common ancestor of hydrophiins and laticaudins, which lived on land, was also estimated to possess a smaller number (205) of intact *OR* genes than other terrestrial snakes (Fig. 3A), it is possible that hydrophiins and laticaudins lost *OR* orthologs in their unique lineages independently. In any case, massive loss of *OR* genes was also confirmed in both hydrophiin and laticaudin lineages after the Hydrophiini–Laticaudini split, coinciding with their transition from land to water. Amphibious carnivorans (pinnipeds and otters) and fully-aquatic cetaceans were also estimated to have lost a large number of intact *OR* genes when they migrated from land to water [21, 24], showing a remarkable case of convergent evolution on becoming aquatic. Although both hydrophiins and laticaudins possess fewer intact *OR* genes, their expression patterns are different from each other. Most of the *OR* genes possessed by *L. laticaudata* are expressed in the NC, while those possessed by *H. melanocephalus* are not (Fig. 4). This contrast indicates that most of the *OR* genes possessed by hydrophiins do not have olfactory function, and that hydrophiins lost a functional MOS. Loss of the MOS in hydrophiins is also supported by the evolution of the *TAAR* genes — the *TAAR2* gene, which is expressed in the NC of laticaudins (Table S3), is pseudogenized in the hydrophiin genomes. Histological studies showed that a relative size of the olfactory region in the NC is highly reduced in sea snakes, and particularly, that of hydrophiins lacks an external nasal gland which lubricates the olfactory epithelium [37, 38]. The role of the MOS is poorly understood in snakes, but these findings suggest that the MOS became less useful for snakes to sense surrounding environment on becoming aquatic, and it was completely lost in fully-aquatic hydrophiins, probably because the snake MOS functions only in the air.

Evolution of the vomeronasal system

The evolutionary pattern of the gain and loss of *V2R* genes differs from that of *OR*s in snakes. Although sea snakes possess a relatively small number of intact *V2R*s, the numbers of intact *V2R* genes vary largely even among hydrophiins and laticaudins (Fig. 2). Snakes are known to have a pair of well-developed vomeronasal organs [34, 37], and fully-aquatic hydrophiins are no exception [37-39]. Most of the intact *V2R*s are expressed in the VNO even in the case of hydrophiins (Fig. 4). The snake VNO is linked to the oral cavity, and snakes deliver odor molecules to their VNOs through tongue-flicking [36]. Underwater tongue-flicking is widely observed among squamates [37] including hydrophiins [37, 40], and *Hydrophis* can distinguish fish species solely by tongue-flicking [41]. All these pieces of evidence strongly suggest that the hydrophiin VNS is functional, and that sea snakes can smell underwater through tongue-flicking. A recent study reported that presence/absence of a *V1R* gene named *ancV1R* corresponds to presence/absence of the functional VNO among tetrapods [33]. We found an intact *V1R* gene orthologous to the *ancV1R* in each snake genome (Fig. S1), and expression of this *V1R* in the VNO is confirmed in hydrophiins (Table S3), supporting the suggestion that the hydrophiin VNS is functional. Gene duplication and loss of the *V2R* genes are more frequent than that of *OR* genes, and massive gain of the *V2R*s is observed even in two species of sea snakes, *E. ijimae* and *L. colubrina* (Fig. 3B). Previous studies showed that *Emydocephalus* relies heavily on the VNS for foraging [40], while not only olfaction but vision plays an important role for *Hydrophis* to find prey [41]. This implies that *Emydocephalus* relies more on olfaction than *Hydrophis*, which is consistent with our results that *Emydocephalus* possesses a larger number of intact *V2R*s than *Hydrophis*. Olfactory capabilities through the VNS may differ largely between snake species including sea snakes.

We found an *OR* gene expressed in the tongue. This gene is conserved among snake species, implying that the function of this OR is evolutionarily maintained and important for snakes.

Unlike most of other *OR* genes, the expression of this gene is confirmed even in hydrophiins (Fig. 4). Snakes do not use the tongue as a gustatory organ because it lacks taste buds [36]. Still, the tongue may be used as a chemosensory organ which enables efficient tongue-flicking of snakes. Further studies are required for testing this hypothesis.

Olfactory capabilities of sea snakes

In this study, we investigated the molecular basis of snake olfaction and showed the presence of the VNS but absence of the MOS in fully-aquatic hydrophiins. Although hydrophiins cannot smell in the air using the MOS, they smell underwater using the VNS. To our knowledge, hydrophiins are the only vertebrates which possess a functional VNS without presence of a MOS. The functional VNS is absent from all extant fully-aquatic mammals (cetaceans and sirenians) though their terrestrial relatives (artiodactyls and terrestrial afrotherians) have it [42], indicating that the VNS is required only on land in most mammals. However, squamates are suggested to smell underwater using the VNS. This may be because *V2R*s, which are abundant in diverse aquatic vertebrates and putatively detect water-soluble molecules, are predominant in squamate genomes over *V1R*s, which have diversified in mammals after their terrestrial invasion to detect odorants on land [43-47]. Modern anurans, particularly in the larval form, also use the VNS for smelling underwater [15] with the VNOs in which *V2R*s are predominantly expressed [47-49].

The olfactory capability of amphibious laticaudins is speculated to be an intermediate between terrestrial snakes and hydrophiins: they still possess a functional MOS, but their *OR* gene repertoire has largely degenerated. This is consistent with our assumption that amphibious species are intermediates between fully-terrestrial and fully-aquatic. Amphibious mammals also tend to show intermediate status between fully-terrestrial and fully-aquatic mammals. For

example, the majority of pinnipeds retain putatively functional VNS, while some species such as harbor seals lack it [50]. Careful interpretation is required for amphibious species when studying convergent evolution among marine amniotes.

Comparison among fully-aquatic amniotes

Our results show that hydrophiins, which adapted to water independently from aquatic mammals, also reduced olfaction profoundly. However, the residual olfactory abilities are very different between fully-aquatic mammals and hydrophiins. Baleen whales smell in the air using a highly degenerated but functional MOS [23, 26]. Little is known about olfactory capacities of sirenians, but they also possess a putatively functional though degenerated MOS [42, 51], and their olfactory anatomy suggests that they smell in the air, not underwater [51]. On the other hand, hydrophiins possess well-developed VNOs, and behavioral studies suggest that they smell underwater using the VNS [40, 41] (Table 1). The well-developed underwater-functional VNS of sea snakes is derived from the V2R-predominant well-developed snake VNS, and the difference of the olfactory capabilities between hydrophiins and fully-aquatic mammals is explained by the difference of the olfactory capabilities between their terrestrial ancestors. Underwater olfaction might have been favored by natural selection if it was adaptive for whales and sirenians to survive in water, but they have never acquired underwater-functional olfactory systems. This shows a striking contrast with the terrestrial adaptation of vertebrates. Tetrapods modified their olfactory organs and generated novel *OR* gene subfamilies for smelling in the air when they migrated from water to land [13, 15]. In addition, adults of secondarily aquatic pipid frogs acquired an additional olfactory epithelium called “water nose” for smelling underwater [47, 52-54]. But no amniotes are known to have modified their olfactory organs for sensing the newly invaded environment upon aquatic adaptation. Histological and genomic studies imply that whales lack innate avoidance behavior against predator odors probably because their

263 predators cannot be detected by smelling in the air [24, 25]. Amniotes belonging to various taxa
264 have to adapt themselves to handle similar problems inflicted by their new environment upon
265 aquatic adaptation. However, not only the ecological demands but phylogenetic backgrounds
266 play important roles in the formation of sensory modalities in this process.

267

268

269 **MATERIALS and METHODS**

270

271 **Specimens**

272 The following specimens were used for DNA/RNA extraction. Specimens used for genome
273 sequencing were not used for RNA extraction in order to save all internal/external organs for
274 future studies. All specimens used in the study are deposited in the Zoological Collection of the
275 Kyoto University Museum (KUZ) with the specimen vouchers shown below.

276 *Laticauda laticaudata* Linnaeus 1758 (blue-lipped sea krait, Laticaudini)

277 1. specimen voucher: KUZ R72402, sex: male, locality: Okinawa Island, Japan (genome
278 sequencing, Fig. S3A).

279 2. specimen voucher: KUZ R68692, sex: female, locality: Okinawa Island, Japan (RNA
280 sequencing).

281 *Hydrophis melanocephalus* Gray 1849 (slender-necked sea snake, Hydrophiini)

282 1. specimen voucher: KUZ R72403, sex: male, locality: Okinawa Island, Japan (genome
283 sequencing, Fig. S3B).

2. specimen voucher: KUZ R73056, sex: female, locality: Okinawa Island, Japan (RNA sequencing).

Laticauda colubrina Schnieder 1799 (yellow-lipped sea krait, Laticaudini)

Specimen voucher: KUZ R77260, sex: male, locality: Ishigaki Island, Japan (genome sequencing).

Emydocephalus ijimae Stejneger 1898 (turtlehead sea snake, Hydrophiini)

Specimen voucher: KUZ R72604, sex: male, locality: Okinawa Island, Japan (genome sequencing).

DNA extraction, sequencing and performing *de novo* assembly

DNA was extracted manually, following the methods of Blin and Stafford [55] with modifications, from muscle tissues of specimens KUZR72402, KUZR72403 and KUZR72604. Whole-genome shotgun (WGS) sequences were generated using Illumina platforms. Paired-end libraries were prepared using the TruSeq DNA PCR-free Sample Prep kit (Illumina) (specimen KUZR72604) and the TruSeq Nano DNA Sample Prep kit (Illumina) (KUZR72402 and KUZR72043). Mate-pair libraries were prepared following Tatsumi *et al.* [56] having a size range of 6-10 kb with a peak of around 7 kb. A PacBio RS II sequencer was also employed for sequencing the *L. laticaudata* genome using the PacBio DNA Template Prep Kit 1.0 (Pacific Biosciences). The details of sequencing results are provided in Table S4, and k-mer frequency spectrum of the WGS reads of each specimen is shown in Fig. S4. Platanus_trim and Platanus_internal_trim [57] were employed to trim low-quality regions and adapters of paired-end and mate-pair sequences respectively with default parameters, except for reads of *L. colubrina*. Regarding PacBio long-reads, following filtering criteria were used to obtain

subreads from the polymerase reads: minimum subread length 50, minimum polymerase read quality 0.75. For the *L. colubrina* (specimen KUZR77260), high-molecular weight (HMW) DNA was extracted from the liver using Nuclei PURE Prep Kit (Sigma). Using the Chromium System (10xGenomics), a linked-read library was constructed from 1.25 ng of HMW DNA of 50 kb or longer. The library was sequenced on an Illumina HiSeq platform, and then processed using Supernova v1.2.2[30, 31] with default settings. Assembling the *L. laticaudata* genome: Trimmed paired-end reads were used to construct contig assembly using the PLATANUS v1.2.4 [57] with a step size of k-mer extension set at 1. Scaffolds were constructed based on this contig assembly using the Redundans v0.12c [58]. Finally, the PacBio subreads were merged, and gap-closing was performed using the PBJelly software in the PBSuite package v15.8.24 [59, 60]. The *H. melanocephalus* genome: PLATANUS v1.2.4 [57] was employed for contig assembling, scaffolding and gap-closing with step size of k-mer extension set to be 1. Only paired-end reads were used for contig assembling, and then mate-pair reads were added for scaffolding and gap-closing. The *E. ijimae* genome: SOAPdenovo2 [61] was employed for contig assembling, scaffolding and gap-closing with a k-mer set to be 81. Completeness of these genome assemblies was evaluated using CEGMA v2.5 [29] and BUSCO v3 [27, 28] referring to the ortholog set CVG [62].

In addition to these sea snake genome assemblies, genome assemblies of terrestrial snakes closely related to sea snakes [king cobra *Ophiophagus hannah* (Elapidae, GenBank Accession GCA_000516915.1), garter snake *Thamnophis sirtalis* (Colubridae, GCA_001077635.2) and common viper *Vipera berus* (Viperidae, GCA_000800605.1)] and a green anole *Anolis carolinensis* (Iguanidae, GCA_000090745.2) were obtained from the GenBank FTP server.

Identification of the olfactory GPCR genes

331 ***OR*:** The *OR* genes were searched against the genome assemblies of seven snake species (four
332 sea snake genomes assembled in this study, and three terrestrial snake genomes retrieved from
333 the GenBank) using the TBLASTN program in the BLAST+ v2.6.0 package [63] with the
334 cutoff *E*-value of 1×10^{-5}. Deduced amino acid sequences of all intact *OR* genes of the green
335 anole and the western clawed frog identified by Niimura [64] were used as queries. Each
336 sequence thus obtained was searched against the NCBI protein database using the BLASTX
337 program and was discarded if its best hit was not an OR. A sequence was judged to be a non-
338 functional pseudogene if the sequence was interrupted by premature stop codon(s) and/or frame
339 shift(s), or it lacked five or more consecutive amino acids including a trans-membrane domain.
340 If a sequence was interrupted by contig-gap(s) although it was not judged to be a pseudogene, it
341 was labeled as 'truncated'. The *OR* gene repertoires of the anole and the python were retrieved
342 from the dataset provided by Vandewege *et al*. [65]

343 ***TAAR* and *V1R*:** Essentially a uniform method employed to find the *OR* genes was used to
344 identify intronless *TAAR* and *V1R* genes from the genome assemblies of seven snakes and a
345 green anole with following amino acid sequences as queries: *TAAR*, all intact mammal TAARs
346 identified by Kishida *et al.* [24]; *V1R*, all intact vertebrate V1Rs identified by Zapilko and
347 Korsching [46].

348 ***V2R*:** Only the longest exon (3rd exon, approx. 800 bp) of *V2R* genes was analyzed in this study
349 because *V2R*s are multi-exon genes and it is difficult to identify all exons derived from a *V2R*
350 gene if exons are scattered in two or more scaffolds. Using deduced amino acid sequences of the
351 3rd exon of all intact vertebrate *V2R* genes identified by Shi and Zhang [43] as queries, *V2R*
352 sequences were searched and identified based on the same method employed to identify *OR*
353 genes.

354

355 **RNA extraction, sequencing and expression analyses**

356 Total RNA was extracted from three potential olfactory organs (the VNO, the NC and the
357 tongue) and the liver (as a negative control) of specimens KUZR68692 (*L. laticaudata*) and
358 KUZR73056 (*H. melanocephalus*) using the RNeasy Mini kit (Qiagen) and following the
359 manufacturer's guidelines. Regarding the NC, we sampled entire tissues around the NC broadly
360 from both specimens for RNA extraction because the olfactory epithelia of both specimens were
361 hard to be located exactly. It is noted that this might cause reduction of the estimated expression
362 level of chemosensory receptors due to contamination of non-olfactory tissues. The extracted
363 RNA was used to construct paired-end sequencing libraries using the TruSeq RNA Sample Prep
364 Kit v2 (Illumina), and these libraries were sequenced using an Illumina HiSeq platform
365 (2×101bp). As a result, the following sizes of RNA-seq reads were obtained: [KUZR68692]
366 VNO 5.19 Gbp, NC 5.27 Gbp, tongue 5.91 Gbp, liver 5.24 Gbp; [KUZR73056] VNO 4.73 Gbp,
367 NC 5.74 Gbp, tongue 5.38 Gbp, liver 8.46 Gbp. Low-quality sequences and adapters were
368 removed using the Trimmomatic v0.36 [66] with the following parameters:
369 ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10, LEADING:20, TRAILING:20,
370 SLIDINGWINDOW:5:25 and MINLEN:36. Trimmed RNA-seq reads were mapped to the
371 conspecific genome assembly using HISAT2 [67] v2.1.0 with default parameters. Gene
372 expression levels were quantified with FPKM values using Cufflinks [68, 69] v2.2.1 after
373 removal of duplicated reads.

374

375 **Phylogenetic analyses**

376 Deduced amino acid sequences were aligned using the L-INS-i program in the MAFFT package
377 v7.266 [70], and gap sites were excluded from further analyses. Trees were inferred using the
378 neighbor-joining method [71] based on the Poisson-corrected distance matrices. Evolutionary

changes in the number of *OR*/*V2R* genes were inferred using the reconciled tree method [72]. Amniote *OR*s are clearly classified into two classes, class I and class II [73]. All intact *OR* genes were classified into 35 clades identified by Niimura and Nei (a class I clade and 34 class II clades) [72] based on sequence similarities, and a calculation was performed for each clade separately. Eight human class I ORs (OR51Q1, OR51G1, OR51L1, OR51I1, OR52K1, OR52H1, OR52B4, OR56A1) retrieved from the HORDE database [74] build #44 were used as outgroups for class II OR trees; 16 human class II ORs (OR1C1, OR1Q1, OR2C1, OR5F1, OR5J2, OR5P3, OR6B2, OR6N1, OR7D4, OR8D2, OR8U1, OR9Q2, OR10A3, OR10K1, OR11H4, OR13D1) for a class I OR tree. Vandewege et al. [65] reported the *OR* gene repertoire of a python, a phylogenetically distant snake species which possesses much larger number of intact *OR* genes (481) than that of any snakes investigated in this study. We included the python intact *OR*s identified by them for this analysis to estimate the ancestral *OR* gene repertoires thoroughly. *V2R* genes were classified into two clades (families C and non-C [44]) based on a phylogenetic tree using green anole Tas1Rs (Tas1R1; GenBank accession no. XM_016998922, Tas1R2; XM_008124605, Tas1R3; XM_003228934) as outgroups, and the evolutionary gains and losses of non-C *V2R*s were calculated using the family-C *V2R*s as outgroups. Because all snakes possess exactly one family-C *V2R*, we concluded that the number of family-C *V2R* did not change through the evolution of snakes. Bootstrap values were obtained by 500 resamplings, and a bootstrap value of 70% was used as a threshold for reconciliation. Truncated genes and pseudogenes were excluded from this calculation. Phylogenetic trees of class I and class II *OR* genes are shown in Figs. S5 and S2 respectively, and changes in the number of class I and class II *OR* genes are shown in Fig. S6.

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Data accessibility

Specimens: Zoological Collection of the Kyoto University Museum (KUZ) with specimen vouchers KUZ R68692, R72402, R72403, R72604, R73056 and R77260. Sequencing data and assembled genome sequences: GenBank BioProject accessions PRJDB7221 (*E. ijimae* genome sequencing), PRJDB7226 (*L. laticaudata* genome sequencing), PRJDB7271 (*H. melanocephalus* genome sequencing), PRJDB7284 (*L. colubrina* genome sequencing), PRJDB7257 (*L. laticaudata* RNA-seq) and PRJDB7258 (*H. melanocephalus* RNA-seq). The locus of each gene (Supplemental Tables S6-S13) and amino acid sequences of intact olfactory GPCRs (Supplemental Data S1) identified in this study: Dryad doi:10.5061/dryad.t8sm4m6 [78].

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Figure captions

Figure 1. A schematic view of the evolution of terrestrial adaptation of vertebrates and three major groups of extant fully-aquatic amniotes. Branch color indicates representative lifestyle in each branch (brown: terrestrial, purple: amphibious, blue: fully-aquatic), and circles in ancestral nodes represent lifestyles at these points in evolution. Extinct amphibious species are also shown for cetaceans (*Amburocetus* [75]) and sirenians (*Pezosiren* [76]).

Figure 2. Phylogenetic relationship of squamates analyzed in this study, and the numbers of olfactory GPCR genes identified in the genome assemblies of these species. Red, pink and grey bars indicate the numbers of intact genes, truncated genes and pseudogenes, respectively. Approximate divergence time follows Sanders *et al*. [4-6] and Kim *et al*. [77]. Notes: *Only the third exon of the *V2R* genes was identified and analyzed. **The *OR* gene repertoire of a green anole is taken from Vandewege *et al.* [65].

671 **Figure 3.** Evolution of the gain and loss of *OR* and *V2R* genes in snakes. Evolutionary changes
672 in the number of intact *OR* (A) and *V2R* (B) genes are estimated using the reconciled-tree
673 method [72]. Python intact *OR*s identified by Vandewege *et al.* [65] were included in the dataset
674 for this calculation.

675 **Figure 4.** Expression levels of the *OR* and *V2R* genes in the three potential olfactory organs and
676 the liver. Each dot represents a single *OR*/*V2R* gene identified in this study, and the y-axis
677 shows normalized gene expression levels in FPKM (fragments per kilobase of exon per million
678 mapped fragments) values. Red, pink and black dots represent intact genes, truncated genes and
679 pseudogenes, respectively. Mean FPKM values of intact, truncated and pseudogenes in each
680 organ are shown as bars in the background. Difference of mean FPKM values of intact *OR*/*V2R*
681 genes between each chemosensory organ and a control (liver) is calculated, and chemosensory
682 organs with obviously (>1) and significantly (p<0.01, paired t-test) larger FPKM values
683 compared to the control are shown with asterisks (see Table S5 for detail). Arrows indicate an
684 intact *OR* gene expressed in the tongue. Approximate position of each organ in a fully-aquatic
685 hydrophiin (*H. melanocephalus*) is also shown.

686

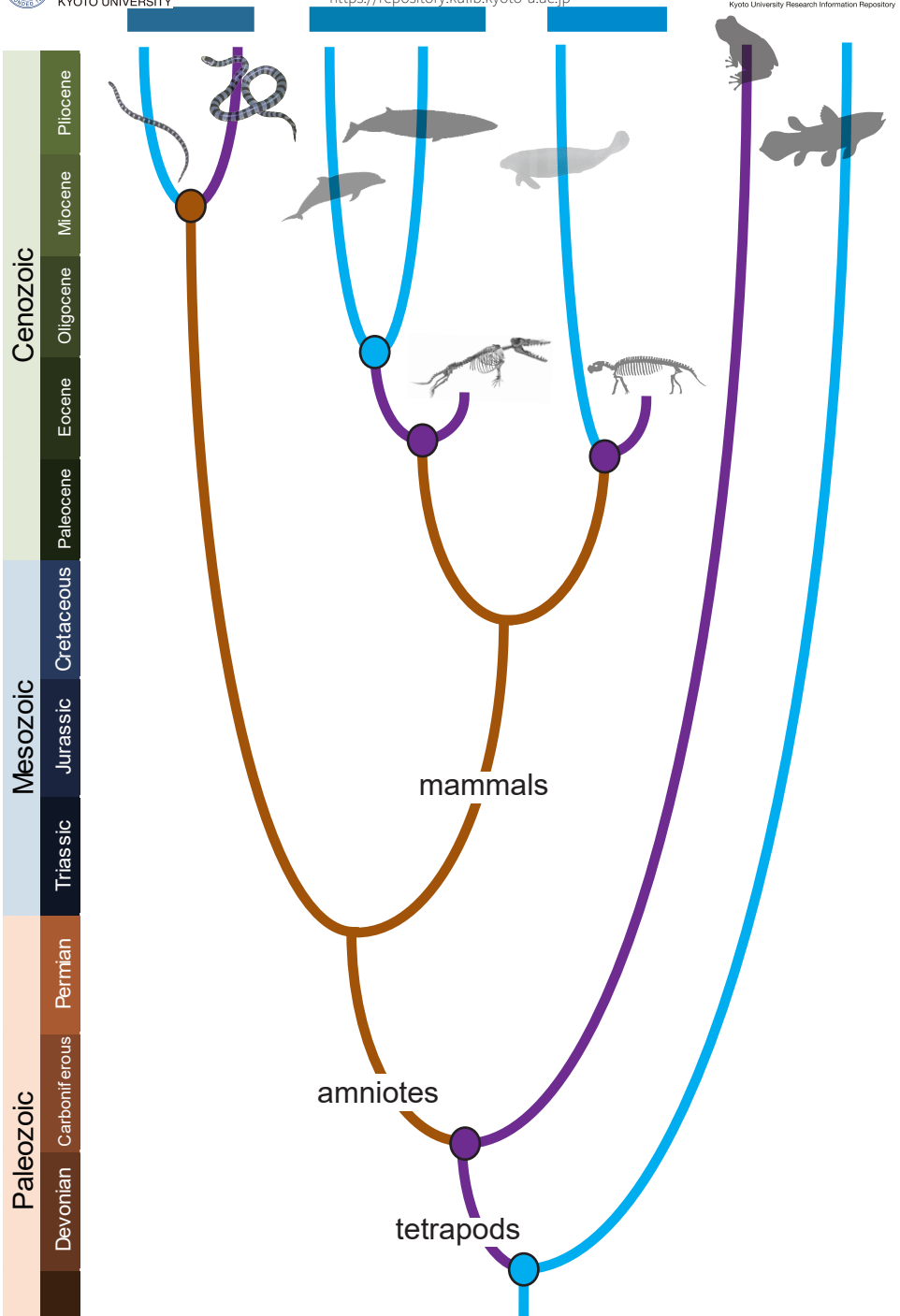
687

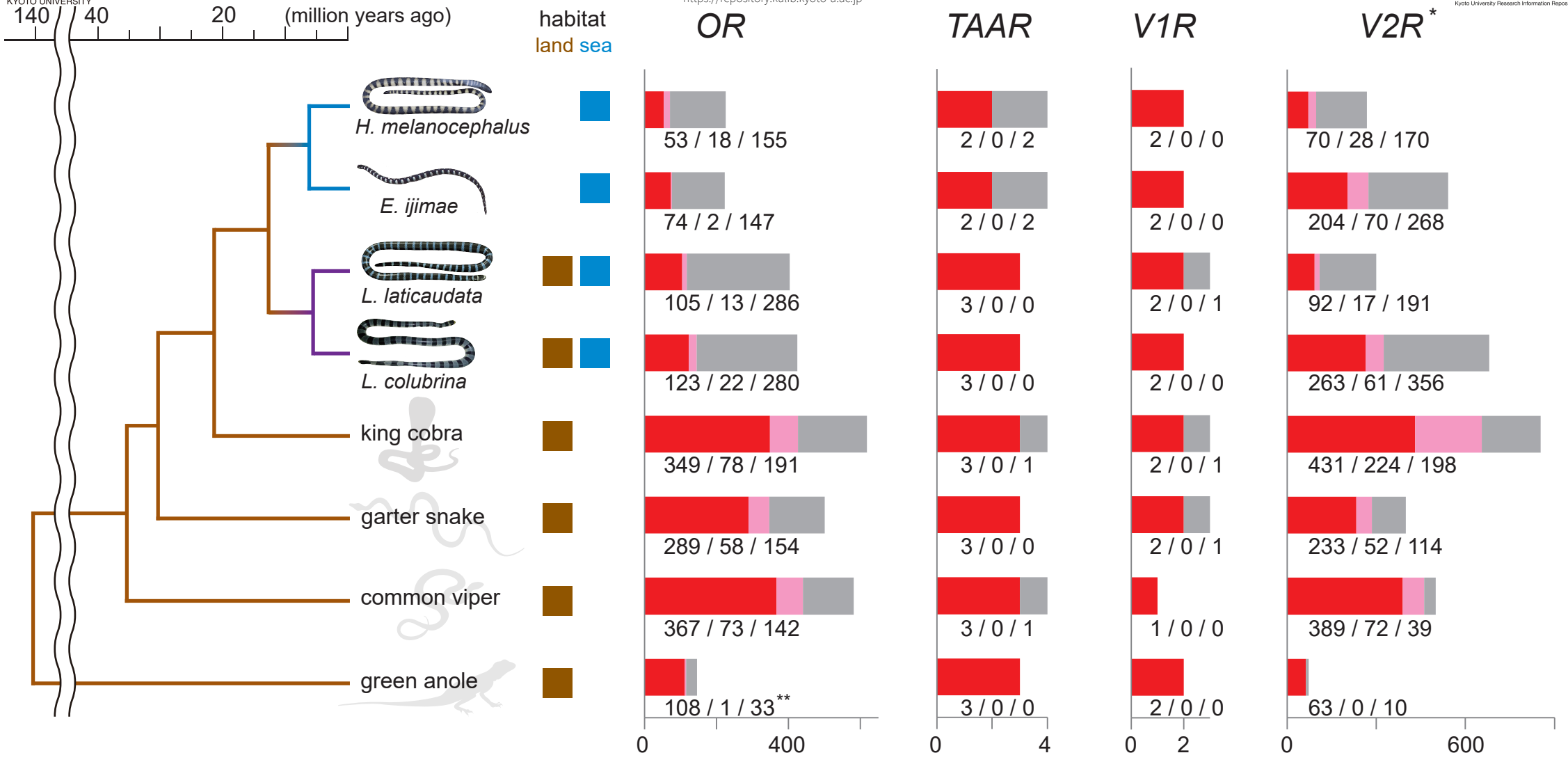
Table 1. Olfactory capabilities of extant fully-aquatic amniotes.

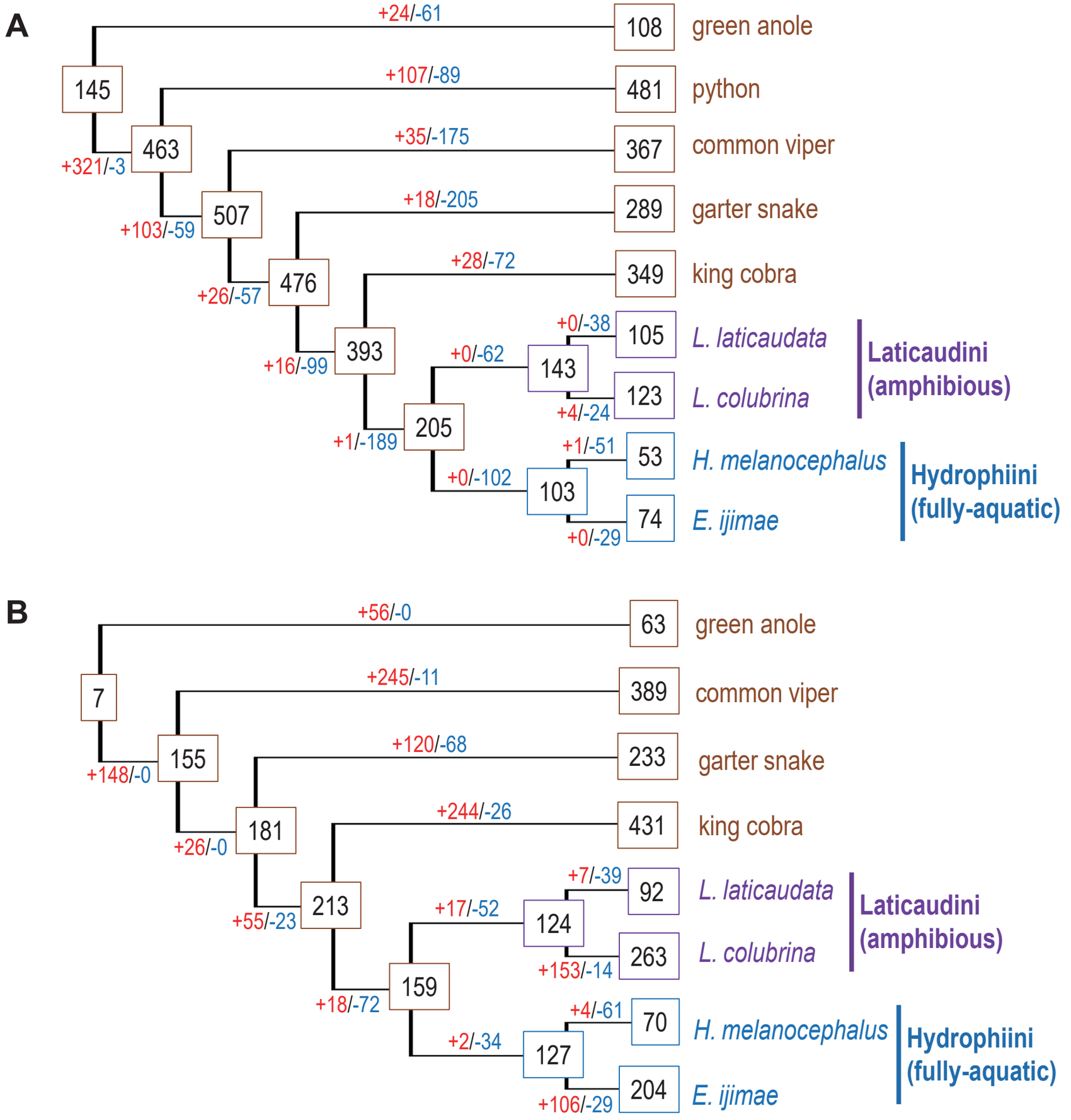
		main olfactory system (MOS)	vomeronasal system (VNS)	references
Cetacea	Odontoceti	absent	absent	[22, 42]
	Mysticeti	present, smelling in the air	absent	[23-26]
Sirenia		present, smelling in the air	absent	[42, 51]
Hydrophiini		absent	present, smelling underwater*	[37-41], this study

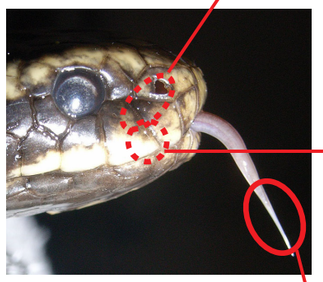
*It remains unknown whether hydrophiins use the VNS for smelling in the air or not.

688









NC

VNO

tongue

liver
(control)

FPKM

0
20
40

*

*

*

OR

V2R

H. melanocephalus
(fully-aquatic)

L. laticaudata
(amphibious)